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Kenneth W. Nickerson
University of Nebraska-Lincoln, knickerson1@unl.edu

Kensal E. Van Holde
Oregon State University

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A Circular Dichroic Study of Cu(II)-Ribonuclease Complexes*

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KENNETH W. NICKERSON‡ AND KENSAL E. VAN HOLDE
From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331

SUMMARY

The visible and ultraviolet circular dichroic (CD) spectra resulting from the interaction of ribonuclease with successive Cu(II) ions have been recorded under a variety of conditions. At pH 7 in the presence of 0.16 M KCl a broad, negative band was found in the visible region. This band increased in intensity and changed in shape as successive coppers were added. The circular dichroic spectra could be analyzed in terms of two kinds of binding sites: a single strong site with CD minimum at about 710 nm, and four weaker sites with CD minimum at about 600 nm. The binding constants observed are close to those obtained by more conventional means. Carboxymethylation of one histidine results in loss of one of the weaker sites. In 0.01 M salt, only the 600-nm band is seen.

Binding at pH 9.6 differed in that saturation did not occur until about 33 sites had been filled. The presence of tetra coordination at this pH was indicated by the shift of the primary d-d transition down to 530 nm. Additional structure in the visible and near ultraviolet CD was now present in the form of a negative band at 355 nm and, for the first two Cu(II)'s added, a positive one at 480 nm.

Strong positive bands were observed at 251 and 305 nm for all pH values ≥ 7. These are tentatively ascribed to charge transfer complexes between Cu(II) and the peptide backbone. The relationship of the Cu(II)-ribonuclease CD spectra to those of natural, copper-containing metalloproteins, both "blue" and "non-blue", is discussed, with special emphasis on the oxyhemocyanins.

A large number of proteins are known to associate with metal ions. These complexes may be divided into metalloproteins and metal-protein complexes (1–3). In metalloproteins the metal is usually found with the protein in the native state, and often a biological function can be ascribed to it. The binding is frequently so strong as to appear irreversible by such procedures as dialysis. On the other hand, the formation of metal-protein complexes involves both specific and nonspecific binding of one or more metal ions to a protein which does not normally require these ions for biological function. The binding constants are characteristically orders of magnitude smaller than those of metalloproteins.

Because the metal-binding sites in metalloproteins so often constitute active or oxygen-binding sites, they have been frequently studied with regard to their specificity of attachment and functional importance. To answer these questions, it is desirable to choose a physical tool which concentrates on those binding sites to the exclusion of the rest of the protein. Where applicable, Mossbauer spectroscopy (4) and electron paramagnetic resonance spectroscopy (3) serve this purpose admirably. An alternative tool is circular dichroism. Circular dichroism possesses a distinct advantage over absorption spectroscopy in that metal ions are optically inactive in solution but may become optically active when bound. One is thus able to look specifically at the ions bound to a protein in the absence of any background contribution from free ions.

This laboratory has previously used CD[1] measurements to study the binding of copper to hemocyanins (5). These results were provocative in that a single visible absorption band was observed to be split into two and three CD bands for arthropod and molluscan hemocyanins, respectively. Furthermore, not only these bands, but CD bands in the ultraviolet (at 350 and 250 nm) were found to be sensitive to the degree of oxygenation. Unfortunately, we were unable to interpret these data in terms of specific amino acid residues at the binding site. Thus, we are attempting to investigate cases in which the copper-binding site is already better characterized, in the hope that such studies will help us to understand the hemocyanin spectra.

The cupric complexes of myoglobin, bovine serum albumin, and numerous peptides have been studied by CD by Gurd and his colleagues (6–8). However, each of these systems has disadvantages as a model for the systems of interest to us. A protein of known conformation is needed, with well defined copper-binding sites and minimal absorption from other chromophores in the near-ultraviolet and visible regions. Ribonuclease seemed an excellent choice (9, 10).

That copper does interact with ribonuclease was initially shown by cupric ion inhibition of both enzymatic activity (11) and active site histidine carboxymethylation (12). Since then the copper-ribonuclease system has been characterized by gel filtration (9, 13), equilibrium dialysis in the presence of a metal ion buffer (14, 15), proton magnetic resonance (16), x-ray crystallography (17), potentiometric titration (18), and chemical modification of the ribonuclease prior to binding (10, 15, 16, 19). These various techniques indicate the presence of up to five

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‡ Present address, USDA-ARS, 1815 N. University, Peoria, Illinois 61604.

[1] The abbreviation used is: CD, circular dichroism.

binding sites at pH values near neutrality. The data can be fitted by the nonunique assumption of one strong and four weak and equivalent binding sites. Much controversy has revolved about the identity of the strong site (9–19). It now seems clear that at pH 7 a site containing in part the α-amino group is filled first (10), followed by four sites involving histidine imidazole nitrogens. The previous uncertainty arose from the extent to which individual site affinities are a function of pH. Binding strength decreases with decreasing pH since the cupric ions must compete with protons for the available protein sites, but the rate of this decrease varies with the type of site involved. By pH 5.5 the α-amino site is no longer the most avid. This was clearly shown by Girotti and Breslow (10) through specific modification of the α-amino group with nitrous acid.

The conclusion drawn is that ribonuclease provides five initial points of attachment for copper, the nitrogen atoms of the α-amino group and the four imidazole rings. This is followed by a varying amount of chelation to the peptide backbone nitrogens. The extent of chelation, and consequently the magnitude of the association constant, depends on the ability of the copper to labilize adjoining peptide hydrogen atoms. This model of ribonuclease-binding sites is based on Sidgwick's observation (20) that copper prefers nitrogen ligands to oxygen ligands, and it gains great support by analogy from Gurd's studies (6–8, 21–23) on small peptides as sufficient models for the interaction of cupric ions with myoglobin and bovine serum albumin. For example, the visible CD spectra of a 1:1 complex of Cu(II) with bovine serum albumin is reproduced almost exactly (6) by a 1:1 complex of Cu(II) with aspartyl-threonyl-histidyl-lysine, the NH_2-terminal tetrapeptide of bovine serum albumin. Similarly, such simple peptides as acetyl-diglycyl-histidyl-glycine have been shown (22) to complex to copper such that they represent an adequate model with regard to titration behavior and visible absorption spectrum for the Cu(II)-sperm whale myoglobin complex.

While circular dichroism has been used as a tool in many of the studies of the relevancy of small peptides as adequate and sufficient models (6–8), no CD spectra have yet been reported for the Cu(II)-ribonuclease complex. We have chosen to study it just because it presents a succession of fairly well characterized copper-binding sites. In addition to providing data for comparison with the CD spectra of metalloproteins, these experiments provide a test for the applicability of the CD method to quantitative binding studies.

MATERIALS AND METHODS

Bovine pancreatic ribonuclease A (code RASE, chromatographically pure, aggregate free) was purchased from Worthington Biochemicals Inc. Any phosphates present were removed by dialyzing five times against distilled water and three times against 0.16 M KCl. Union Carbide cellophane casing was used. Ribonuclease concentrations were routinely determined spectrophotometrically, assuming 1 mg per ml to yield an absorbance at 280 nm of 0.695 (24). A stock solution of 20.7 mg per ml in 0.16 M KCl was diluted as needed.

The carboxymethylated derivative of ribonuclease was prepared by the method of Crestfield *et al.* (25). One hundred milligrams of phosphate-free ribonuclease in 8 ml were brought to pH 5.5, at which point 1.2 ml of iodoacetic acid (10 mg per ml), already at pH 5.5, were added. After 9 hours at room temperature in a pH-stat, the reaction mixture was placed on a Bio-Rex 70 column (1 × 10 cm) (100 to 200 mesh) equilibrated with 0.1 N acetic acid. The column was washed with 60 ml of 0.1 N acetic acid and the ribonuclease eluted with 40 ml 1.0 N sodium acetate. The eluate was dialyzed against 4 liters of distilled water, followed by 1 liter of 0.16 M KCl. No attempt was made to separate ribonuclease carboxymethylated at histidine-12 from that at histidine-119, and the extinction coefficient was assumed to remain unaltered. To check that only the desired histidine carboxymethylation reaction had occurred, a lyophilized sample was hydrolyzed for amino acid analysis. The results differed from those of authentic ribonuclease only by the presence of three rather than four histidines. It was also confirmed that the CD spectrum above 240 nm, an index of protein tertiary structure, remained identical with unmodified ribonuclease.

The basic technique by which we followed Cu(II)-ribonuclease binding with circular dichroism is as follows. About 27 ml of unbuffered ribonuclease solution were prepared by dilution of the stock. To this solution aliquots of 0.04 M $CuCl_2 \cdot 2H_2O$ were added. The pH was adjusted after each addition of $CuCl_2$ by a Radiometer type TTT1 pH-stat filled with "Acculute" 0.2 N NaOH from Anachemia Chemicals Ltd. Depending on the ribonuclease concentration present, 20- or 100-μl Eppendorf pipettes were used to add the stoichiometric amounts of $CuCl_2$. After each addition and pH adjustment the sample was transferred to a 10-cm cylindrical cuvette and the circular dichroism spectrum recorded at room temperature on a Durrum-Jasco model CD-SP apparatus. This procedure was repeated as often as required. A jacketed, constant temperature cell was used occasionally to insure that room temperature variations over 22–28° were without effect. The number of protons released due to binding was estimated from the amount of NaOH needed to restore a given pH after each aliquot of $CuCl_2$ had been added. Our values were very similar to those observed by Breslow and Girotti (18).

In all of the CD spectra reported here the ordinate is the observed ellipticity, θ. However, when CD intensities are compared on a per copper basis the molecular ellipticity $[\theta]$ in deg·cm^2 per dmole is used, where $[\theta] = \theta\ M/10\ l\ c'$. Here θ is the observed ellipticity in degrees, l the path length in cm, c' the concentration in g per cm^3, and M the gram molecular weight of the protein. It should be noted that these are not residue ellipticities.

There has been some evidence (9, 13) that copper binding may be accompanied by association of the ribonuclease to dimers or higher aggregates. Accordingly, average molecular weights were determined by sedimentation equilibrium after the addition of up to 8 copper eq. A Spinco model E analytical ultracentrifuge with ultraviolet absorption optics and scanner was used. All solutions for these experiments were 1 mg per ml in 0.16 M KCl at pH 7.

RESULTS

Binding at pH 7.0—The visible CD spectra observed when successive equimolar increments of $CuCl_2 \cdot 2H_2O$ were added to ribonuclease in 0.16 M KCl are shown in Fig. 1. A single broad band is observed; that it may be composite is suggested by the fact that the wave length (λ_{min}) of the CD minimum shifts with addition of more copper (Fig. 1 and Fig. 2, *Curve A*). The increase in the negative CD value at a representative wave length (600 nm) is shown in Fig. 3. Initially, we thought that the leveling off observed at high Cu:RNAse ratios indicated that saturation had been reached. Now, for reasons given below, we feel that this is not the case, and that the lower value observed at 9 eq is spurious.

The variation in λ_{min} with addition of copper (Fig. 2) suggests

that we are in fact observing a spectrum with contribution from more than one CD band. This might be expected from earlier studies (10) which indicated that under these conditions copper binds to two kinds of sites: an NH_2-terminal site with a binding constant of 5.0×10^5 (moles per liter)$^{-1}$, and four histidine sites with roughly equal constants of about 1.9×10^4 (moles per liter)$^{-1}$. The wave length variation in *Curve A* of Fig. 2 suggests that the stronger site must give rise to a CD band centered near 700 nm and the weaker site to a band centered below 620 nm. Accordingly, we attempted to resolve the observed CD spectra, using a DuPont curve resolver, into two such bands. It was found that all of the spectra shown in Fig. 1 would be accurately fitted by combination of two bands of fixed wave length and breadth but of varying intensity. One of these is centered at 710 nm and the other at 600 nm.

The band at 710 nm reaches saturation at quite low Cu:RNAse ratios (Fig. 4). Therefore, we have identified it as corresponding to the strong, NH_2-terminal binding site. The 600-nm band, which ultimately becomes much more intense, has been assigned to the four histidine sites. We then ask whether the data are consistent with the binding constants obtained by Girotti and Breslow (10). We have approached this question in the following ways. Given the binding constants and num-

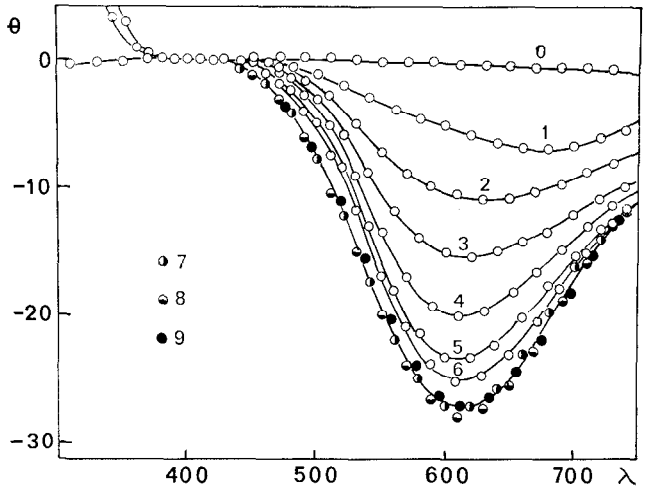

FIG. 1. Visible CD spectra of ribonuclease at 0.8 mg per ml with the addition of 9 successive equivalents of Cu(II). Ten-centimeter path length, pH 7.0, no buffer, 0.16 M KCl. Ellipticity in millidegrees.

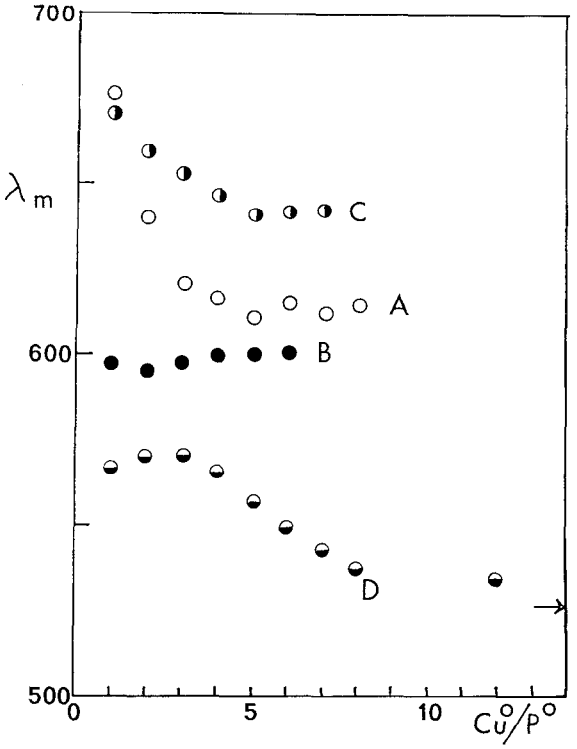

FIG. 2. Wave length of the visible CD minimum *versus* equivalents of Cu(II) added per mole of ribonuclease. *A* (○), data from Fig. 1 (pH 7.0, no buffer, 0.16 M KCl); *B* (●), data from Fig. 6 (pH 7.0, no buffer, 0.01 M KCl); *C* (◑), data from Fig. 7 (pH 7.0, 0.05 M Tris buffer); *D* (◒), data from Figs. 10 and 11 (pH 9.6, no buffer, 0.16 M KCl).

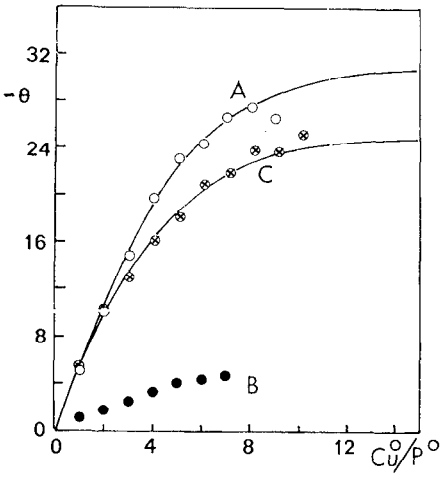

FIG. 3. Ellipticity at 600 nm *versus* number of equivalents added per mole of ribonuclease. All data are normalized to a ribonuclease concentration of 0.8 mg per ml in a 10-cm cell. *A* (○), data from Fig. 1 (pH 7.0, no buffer, 0.16 M KCl); *B* (●), data from Fig. 7 (pH 7.0, 0.05 M Tris buffer); *C* (⊗), data from carboxymethylated ribonuclease under conditions identical with Fig. 1. The curves drawn through *A* and *C* are calculated from the binding constants given by Girotti and Breslow (10), as described in the text.

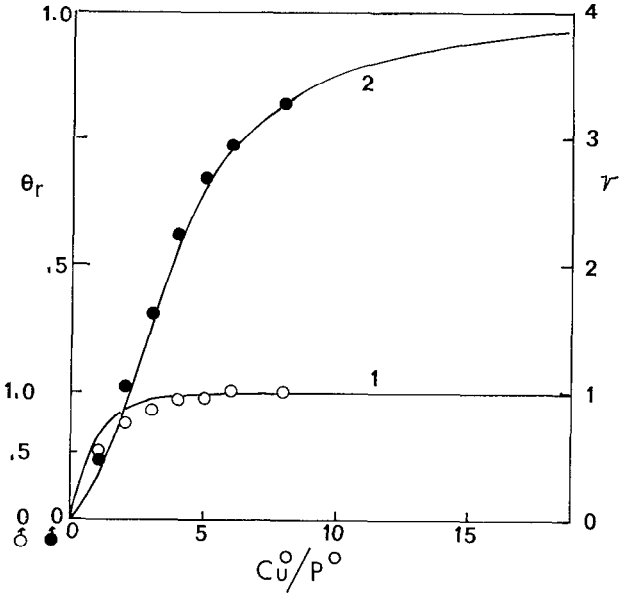

FIG. 4. A graph in which the binding curves for types 1 and 2 sites (*solid lines*, right ordinate) calculated from Girotti and Breslow's binding constants, are superimposed on relative CD intensities (*points*, left ordinates) for the 710- and 600-nm bands.

bers of groups involved, one may calculate, for a given value of free copper concentration [Cu], the binding numbers (ν_1 and ν_2) for the strong and weak sites respectively:

$$\nu_1 = \frac{n_1 K_1[\mathrm{Cu}]}{1 + K_1[\mathrm{Cu}]} \quad (1)$$

$$\nu_2 = \frac{n_2 K_2[\mathrm{Cu}]}{1 + K_2[\mathrm{Cu}]} \quad (2)$$

In this case, we consider $n_1 = 1$ and $n_2 = 4$. The total copper concentration is given by ($[\mathrm{Cu}] + (\nu_1 + \nu_2)P^0$), where P^0 is the total molar protein concentration. This allows construction (Fig. 4) of graphs of ν_1 and ν_2 *versus* Cu^0/P^0, where Cu^0 is the total copper concentration. Comparison of the data with the theoretical curves (Fig. 4) indicates that the bands at 710 and 600 are measuring binding by the two kinds of sites in good agreement with the results of Girotti and Breslow.

A more direct test is possible. If one rewrites Equation 2 in terms of the total copper concentration (Cu^0) one obtains:

$$\nu_2 = \frac{n_2 K_2(\mathrm{Cu}^0 - (\nu_1 + \nu_2)P^0)}{1 + K_2(\mathrm{Cu}^0 - (\nu_1 + \nu_2)P^0)} \quad (3)$$

If a single CD band can be attributed to the type 2 sites, we may define the "reduced" dichroism of this band (θ_r) as the ratio of the observed dichroism to the saturating value. Then $\nu_2 = n\theta_r$, and Equation 3 can be rearranged to:

$$\frac{\theta_r}{1 - \theta_r} = nK_2P^0\left[\frac{\mathrm{Cu}^0}{nP^0} - \theta_r - \frac{\nu_1}{n}\right] \quad (4)$$

A graph of $\theta_r/(1 - \theta_r)$ *versus* the quantity in brackets on the right of Equation 4 is shown in Fig. 5. The slope of the straight line yields a value of $K_2 = 2.3 \times 10^4$ (moles per liter)$^{-1}$, in reasonable agreement with the value of 1.9×10^4 (moles per liter)$^{-1}$ obtained by Girotti and Breslow (10). Comparable calculations for the type 1 sites have not been attempted, since the type 2 binding so completely dominates the behavior. However, as can be seen from Fig. 4, the first site, by our measurements, behaves approximately as expected from the data of Girotti and Breslow.

The data shown in Fig. 3 can also be interpreted in the same manner. The curve drawn through the points is predicted from the values of ν_1, ν_2 calculated from Girotti and Breslow's data. If θ_1 and θ_2 represent the contributions per bound copper to the observed ellipticity at 600 nm, then:

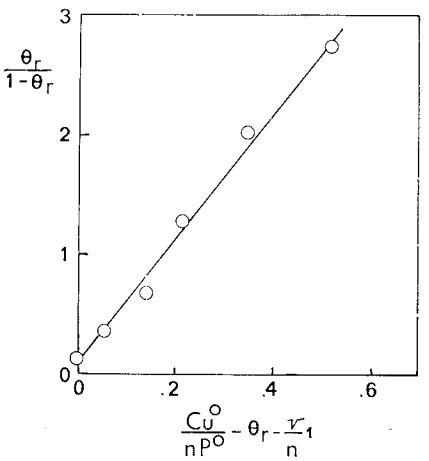

FIG. 5. A graph to evaluate the binding constant for the four weak (type 2) sites, according to Equation 4. In the calculation, ν_1 was taken from our data in Fig. 4. See the text.

$$\theta = \nu_1\theta_1 + \nu_2\theta_2 \quad (5)$$

Values of θ_1 and θ_2 can be obtained by simultaneous solutions of Equation 5 at two points. We utilized the value of θ at $\mathrm{Cu}^0/P^0 = 1$, at which point the data of Girotti and Breslow indicate $\nu_1 = 0.66$ and $\nu_2 = 0.28$, together with the limiting value, where $\nu_1 = 1$ and $\nu_2 = 4$. The limiting value was chosen by trial and error to give the best fit. The ratio of this limiting value to the largest ellipticity measured was the same as the corresponding ratio used to define θ_r in Equation 4.

While this method of fitting the data leaves much to be desired, it is supported by a comparable analysis of the results for carboxymethylated ribonuclease (Fig. 3). The curve drawn through these points utilized the *same* parameters θ_1 and θ_2, the *same* binding constants, but the assumption that one strong site and only *three* histidine sites were present. The agreement is moderately good.

Taken together, these results strongly support the contention of Girotti and Breslow that in 0.16 M KCl, pH 7.0, ribonuclease possesses one strong site and four weaker sites for copper binding. Our results go somewhat further, however, in that the linear relation observed in Fig. 5 indicates that the weaker sites are, in fact, nearly equivalent and that they do not exhibit strong cooperativity. Extreme non-equivalence or cooperativity should lead to non-linearity in such a graph. While strict equivalency is not to be expected, in view of the different environments of the histidines, whatever differences exist are not detectable by our methods.

Part of the reason the identity of the single, strong site has created so much controversy (9–19) is that it is not uniformly the most avid site. This is known to be true if the pH is lowered to 5.5 (10, 16). Fig. 6 shows that it also appears to be the case at pH 7 if the ionic strength is lowered from 0.16 to 0.01. Under these conditions, the wave length of maximum ellipticity is constant, and at a bit below 600 nm (Fig. 2*B*). If we assign the 710-nm CD band to the first site occupied at high salt, we are forced to conclude that either (*a*) that site is no longer active at low ionic strength, possibly because of electrostatic repulsion between Cu^{++} and the doubly charged NH_2-terminal lysine; or (*b*) the nature of the binding at the NH_2-terminal site has been modified such that it too now has a 600-nm band. Unfortunately, our data in 0.01 M salt are not complete enough to yield an unambiguous answer to this question.

The data in Fig. 6 have been extended to cover the range from 300 to 800 nm. At the low wave length end we find the positive band hinted at in Fig. 1 more clearly expressed. It too must be a copper band, for ribonuclease alone has no absorption or CD in this region. The spectra were extended to 800 nm here to assure that we were looking at all of the Cotton effects resulting from copper d-d transitions. If copper is present in its preferred, Jahn-Teller distorted, square planar configuration, it is supposed (3) to give a maximum of three transitions. However, four can be observed under less symmetrical conditions. Yasui *et al.* (26) report four d-d Cotton effects for complexes of Cu(L-serine)$_2$, with the last one at 830 nm. We have no clear indication of any further bands in this region.

Fig. 7 shows data obtained at pH 7.0 when 0.05 M Tris buffer was used to stabilize the pH. Since Tris is also known to bind Cu(II) (27), the concentration of free Cu(II) is kept at levels very much lower than would be indicated by the equivalents of $CuCl_2$ added. Thus, only the most avid sites would be expected to be filled. The data of Fig. 7 are also graphed in Figs. 2 and 3. A glance at Fig. 3 suggests that only the single,

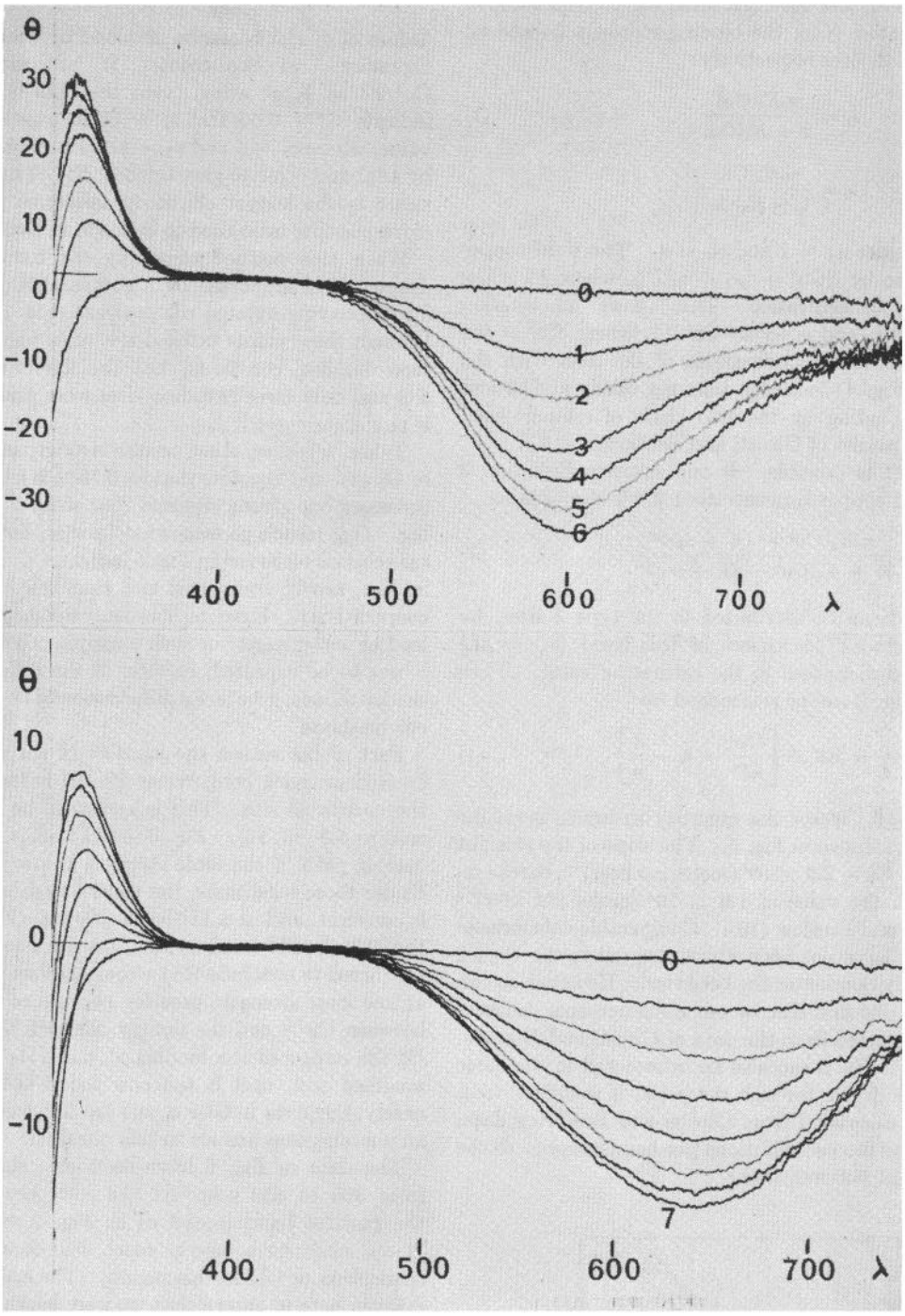

FIG. 6 (*top*). Visible and near ultraviolet CD spectra of ribonuclease at 1.2 mg per ml with the addition of 6 successive equivalents of Cu(II). Ten-centimeter path length, pH 7.0, no buffer, 0.01 M KCl. Ellipticity in millidegrees.

FIG. 7 (*bottom*). Visible and near ultraviolet CD spectra of ribonuclease at 2 mg per ml with the addition of 7 successive equivalents of Cu(II). Ten-centimeter path length, pH 7.0, 0.05 M Tris buffer present for this experiment only. Ellipticity in millidegrees.

strongest site is being occupied at the Cu(II) concentrations achieved in Tris. However, Fig. 2 shows that the situation is more complex. Here too a shift in the wave length of CD minimum occurs, and the resultant band position indicates that when 7 eq of Cu(II) have been added, the 710-nm site and at least one of the 600-nm sites are about equally occupied. We believe this situation represents an intermediate stage between that found at 0.16 and 0.01 ionic strength. The 710-nm site is still the strongest, but the margin by which its affinity is greater than the 600-nm sites has decreased. Thus, with decreasing ionic strength, the 710-nm site binds more weakly.

Finally, we must raise the question whether changes in the conformation or state of aggregation of ribonuclease, or both, occur as a consequence of Cu(II) binding at pH 7. Fig. 8 presents some data relevant to the first question. It is evident that no appreciable change in CD over the range from 215 to 240 nm is caused by the addition of up to $6\frac{2}{3}$ eq of Cu(II). This may be taken as presumptive evidence that the secondary

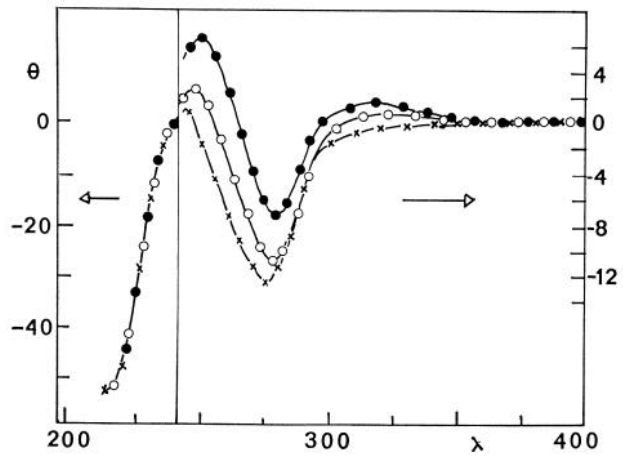

FIG. 8. Far ultraviolet CD spectra of ribonuclease at 0.6 mg per ml with two additions of 3⅓ eq of Cu(II) each. pH 7.0, 0.01 M KCl, path length = 1 cm above 240 nm and 1 mm below. ×——×, no copper added; ○——○, 3⅓ coppers added; ●——●, 6⅔ coppers added. Note change in scale below 240 nm. Ellipticity in millidegrees.

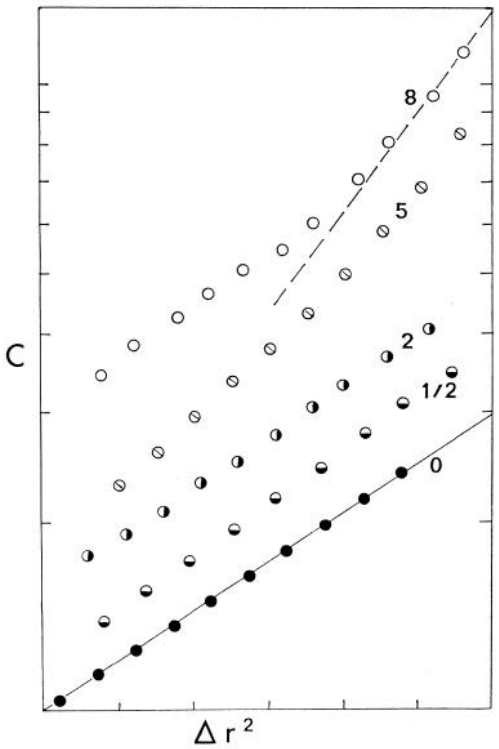

FIG. 9. Sedimentation equilibrium of 1 mg per ml of ribonuclease in 0.16 M KCl at pH 7.0. The number of Cu(II) equivalents added to each is shown by the adjoining numeral. Data were taken with the ultraviolet scanner at wave lengths near 280 nm. All data are at 20,000 rpm and 22°. The set of points have been arbitrarily displaced on the log c axis. The abscissa gives the square of the distance from the meniscus. The *solid line* through the O-Cu set of points is that predicted for molecular weight 13,683. The *broken line* is of the slope predicted for a ribonuclease dimer.

structure is unchanged. The situation in the 240- to 300-nm region is more complex. Changes are evident, but we feel they can be largely accounted for by the emergence of two positive, charge-transfer bands involving copper. The one at 305 nm has already been mentioned, and the one at 251 nm will be discussed in detail later. With these provisos, there is no definite evidence

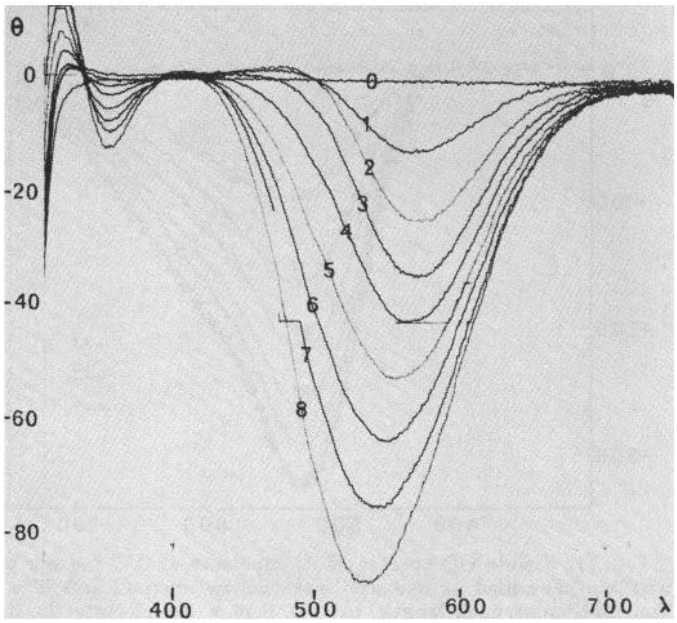

FIG. 10. Visible CD spectra of ribonuclease at 0.8 mg per ml with the addition of 8 successive equivalents of Cu(II). pH 9.6, no buffer, 0.16 M KCl, 10-cm path length. The *short horizontal lines* result from the recorder pen sticking. The number of equivalents added is given beside each curve. Ellipticity in millidegrees.

for a change in CD due to the aromatic amino acids as a consequence of Cu(II) complexing. However, small changes would be difficult to detect.

From gel filtration studies, other workers (9, 13) have suggested that ribonuclease undergoes association on Cu(II) binding. We have carried out a few sedimentation equilibrium experiments at pH 7.0 and 0.16 M KCl. Although association obviously does occur, our scanner data are not especially precise and we do not feel they warrant a detailed analysis. The log c *versus* r^2 graphs are presented in Fig. 9. They clearly show that until more than 2 eq of Cu(II) have been added the material remains homogeneous, with a molecular weight very close to that expected for monomeric ribonuclease. The *solid line* passing through the 0-Cu control of Fig. 9 corresponds to a molecular weight of 13,683. Some aggregated material is evident in the samples to which 5 and 8 eq of Cu had been added. Their limiting slopes do correspond approximately to that expected for dimer (*broken line*), but the solutions are heterogeneous. This might be explained most simply by assuming that the association is reversible, although that point is certainly not proven by our data.

The most interesting point is that absolutely no association is seen until more than two coppers have been bound. It is reasonable to expect the NH_2-terminal site, which is filled preferentially, not to be involved in association since the lysine can form an intramolecular chelate. Occupation of one or more of the histidine sites must then confer the ability to dimerize. In connection with this, it should be noted that histidine-105 is known to be a surface site (28) and might therefore be involved in dimerization.

Binding at pH 9.6 and 5.5—It is evident from Figs. 10 and 11 that the CD spectra observed when Cu(II) is bound to ribonuclease at pH 9.6 are more complex than those found at pH 7.0. The positive band at 320 nm seems invariant (note that it is truncated by the negative aromatic bands and is probably

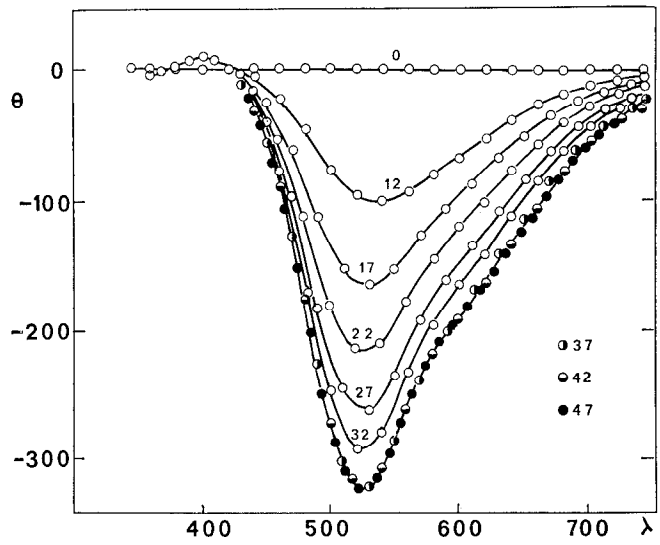

FIG. 11. Visible CD spectra of ribonuclease at 0.77 mg per ml with Cu(II) added at five-step intervals between 12 and 47 eq. Ten-centimeter path length, pH 9.6, 0.16 M KCl. Note the 37, 42, and 47 curves totally overlap. Ellipticity in millidegrees.

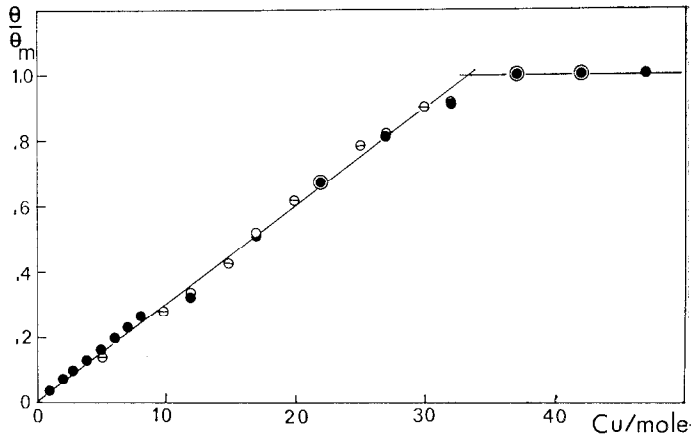

FIG. 12. Data of Figs. 10 and 11 and several similar experiments graphed to show the ellipticity at the visible minimum divided by the maximum ellipticity value obtained (saturation value), at a variety of Cu(II) concentrations up to 47 eq of Cu(II) added. Different symbols represent separate experiments. In all cases they are pH 9.6, no buffer, 0.16 M KCl.

centered at about 305 nm), whereas the strong, negative band in the visible has been shifted down to a much lower wave length. The pH 9.6 CD spectra also reveal a new negative band at 355 nm and, for the first few Cu(II)'s bound, a weak, positive band around 470 nm. The interpretation of these spectra is aided by comparison with the high pH CD spectra of model peptides (6–8). At these high pH values Cu(II) is capable of labilizing sufficient peptide protons to allow tetradentate liganding to the protein (6–8, 21–23). Thus, a histidine-containing site could have copper liganded by an imidazole nitrogen and three peptide nitrogens. The CD spectra of some model Cu(II)-tetrapeptide complexes that contain histidine (6, 7) closely resemble the curves shown in Fig. 10 for the first few coppers added in that they both have a positive band at 470 to 500 nm and a negative one at 560 to 600 nm. This indicates that a portion of the first copper ions added at pH 9.6 go into such histidine-containing sites. When the data of Fig. 10 are plotted in Fig. 2, it is seen that the wave length of the CD minimum remains relatively constant at 570 nm for the first four Cu(II)s added before falling off. When Cu(II) is tetraliganded, in non-histidine-containing model peptides (by three peptide nitrogens and an α-NH_2), a single CD minimum in the 515 to 560 nm range is observed (6–8). Thus, both the NH_2-terminal site and sites involving four peptide nitrogens should be expected to absorb in this region at high pH. Figs. 2, 10, and 11 show that after the four presumed histidine-containing sites on ribonuclease have been filled, the addition of further Cu(II)s causes a shift toward wave length minima characteristic of such totally peptide liganding.

The enhanced lability of the peptide protons at pH 9.6 makes many more sites now available. Just how many can be seen in Figs. 11 and 12. The increase in CD intensity with added Cu(II)'s saturates very sharply at about 33 $\pm$ 3 copper ions added. Because of errors which are bound to accumulate with so many transfers and pH adjustments, the exact number is somewhat imprecise, but it is clear that after a certain point the addition of 10 more copper eq produces zero further CD change. This limit of 33 copper-binding sites corresponds very nicely to that expected if almost all of the peptide nitrogens were involved. Ribonuclease contains 124 peptide backbone nitrogens, four imidazole rings, and 10 lysine ϵ-amino groups. Together these would give about 34 tetradentate-binding sites. The possible participation of the six tyrosine and nine carboxyl side chains might provide about four more. In any event, it is clear that the protein must unfold completely to provide so many sites. In accordance with this requirement that the native secondary structure must be lost, we have found that in this case the 215 to 240 nm CD is drastically decreased, as expected. We may expect that this phenomenon is general for proteins since it is in essence the biuret reaction.

Whereas the visible CD band was used in Figs. 11 and 12 to determine that pH 9.6 saturation occurred at 33 Cu(II)-binding sites, it should be pointed out that other bands of interest are present also. Fig. 13 shows a scan at pH 9.6 from 240 up to 750 nm in the presence of saturating amounts of Cu(II). The band at 251 nm clearly dominates the spectrum.

Hartzell and Gurd (8) have used CD to study the copper complexes of nonaromatic pentapeptides. They consistently observed both a positive band at 305 nm, which corresponds nicely to that we have found truncated at 320 nm, as well as a 275-nm band. They attributed these bands to charge-transfer complexes between copper and the deprotonated peptide backbone. We have no indication of a 275-nm band, but we believe our dominant 251-nm CD band represents a comparable charge-transfer band. It is about six times as intense as the visible copper CD band at 525 nm. Although there is no exact correlation between the intensities of CD and absorption bands, this agrees rather well with the generalization (1, 3) that charge-transfer bands are about 10 times more intense than d-d transitions. The 251-nm band must be characteristic of a great variety of binding sites. This is a reasonable expectation for the deprotonated peptide backbone.

The positive CD bands at 251 and 305 to 320 nm are unique among those we have observed in that their position is not sensitive to pH, showing very little change between pH 7.0 and 9.6. This insensitivity of λ to changes in environment above pH 7 was also noted for the 305-nm band by Hartzell and Gurd (8). Their use of peptides containing no aromatic residues allowed them to study the latter band at 305 nm instead of truncated at 320 nm. It also completely eliminates the possibility that the 305-nm band is derived from an ionized tyrosine. In our experiments, the position and shape of the 251- and 320-nm

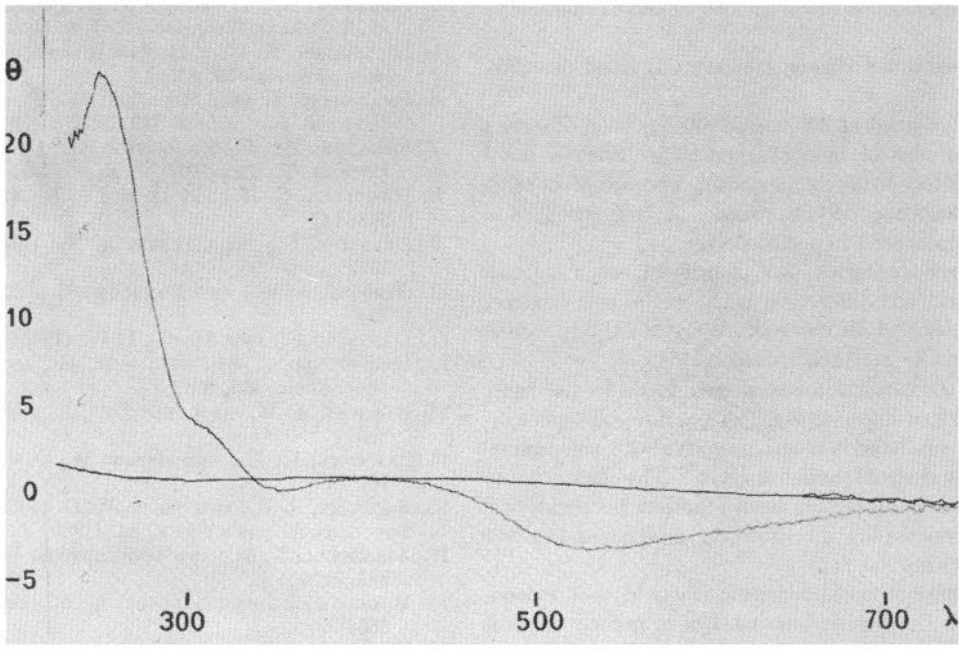

FIG. 13. Visible and ultraviolet CD spectrum of ribonuclease at 1 mg per ml with 37 eq of Cu(II) added. One-millimeter path length, pH 9.6, no buffer, 0.16 M KCl. Ellipticity in millidegrees.

bands were invariant with numbers of copper added at pH values of 7.0 or 9.6. This means that tetra coordination is not a prerequisite. Here the charge-transfer bands differ from both the positive 470-nm histidine band and the negative 355-nm band, both of which are only present at pH 9.6 (Fig. 10). In these cases tetra coordination appears to be necessary. The 355-nm band is very close to known bands in hemocyanin (5) and other copper-containing metalloproteins (3) that have often been attributed (3) to copper dimer formation. This is thought to be in a manner similar to that found in many polynuclear copper complexes.

In conclusion, we will make brief mention of our attempted studies on copper binding at pH 5.5. We have not been able to compare our results with those of others, either in crystal (17) or solution (9, 10, 13–16, 18) since we get virtually no CD ellipticity at this pH. This is unfortunate since so much of the previous work was concentrated here, but it is to be expected since any binding that does occur at pH 5.5 should be unidentate. The copper is not yet able to labilize an adjoining peptide backbone proton. No protein chelate is formed. We are measuring optical activity, and only when the added copper forms a chelate, as it does at pH 7 or above, do we get a strong signal. The weak ellipticity we do observe at pH 5.5 is broadly centered above 700 nm, which would agree with the suggestion (10, 16) that copper is bound to a single imidazole. This situation is almost identical with that observed for pH 5.5 complexes of Cu(II) with model peptides (8). Our data are essentially negative. If CD were the only criterion of binding, we would have little indication that binding had actually occurred. Similarly, in the aromatic (240 to 300 nm) and peptide (215 to 240 nm) regions no differences at all result from the addition of $6\frac{2}{3}$ eq of Cu(II). This is further indication that low pH binding must be unidentate. The strong 251-nm band resulting from copper binding to the peptide backbone is totally absent.

DISCUSSION

We are now in a position to compare our CD results on ribonuclease with those previously obtained (5) for the oxyhemocyanins. Metalloproteins are known to differ from metal-protein complexes in that they are characterized by tight binding (K_{diss} is often $\leq 10^{-9}$ M (1–3)) and large visible extinction coefficients; that for octopus oxyhemocyanin is 500 liters per g·atom copper cm at 570 nm (29). In contrast, the Cu(II)-ribonuclease system appears to be typical of metal-protein complexes in that (at neutral pH) its values are only about 10^{-4} and 85, respectively (18). Both affinity for copper and the resultant visible extinction coefficient are known to increase as the solution becomes more alkaline, but even at pH 11, when maximum coordination has been achieved, the visible absorption is still only one-third that of a true copper-containing metalloprotein such as oxyhemocyanin. These differences become even larger when we consider that for a copper-containing metalloprotein, oxyhemocyanin's visible absorption is weak enough for it to have been classified as a "non-blue" copper protein (3). Certain of the "blue" copper proteins absorb 10 times more intensely, again at neutral pH.

In this light, it is not too surprising to find that whereas visible CD intensities for oxyhemocyanins range from 2300 to 3000 deg·cm² per dmole (5), that for the first copper added to ribonuclease at neutral pH (Fig. 3) is only about 1000 deg·cm² per dmole. On a per copper basis these values become more comparable at pH 9.6, by which time the Cu(II)-ribonuclease ellipticity has risen to about 2000 deg·cm² per dmole. These values approach those of true copper metalloproteins more nearly than do the extinction coefficients referred to earlier, but they are still decidedly less.

What is remarkable, however, is the definite qualitative resemblance between the alkaline CD spectrum of Cu(II)-ribonuclease and that exhibited by the oxyhemocyanins at both neutral and alkaline pH. The following similarities should be noted.

1. In both cases visible CD bands are observed at 450 to 500 nm and between 500 and 600 nm. In particular, the hemocyanin CD spectra resemble those of histidine-containing peptides and ribonuclease at low levels of added copper.

2. A negative band around 350 nm is common to both, al-

though its relative intensity is much greater in the oxyhemocyanins.

3. Both systems exhibit a strong, positive CD band near 251 nm.

4. A weak, negative band at 305 to 310 nm has been observed in deoxygenated samples of hemocyanins (30). Such a band would be hard to detect in oxyhemocyanin, because of overlap from the intense, negative 350-nm band. A suggestion of a shoulder near 305 nm is seen in some spectra.

Although the above similarities are of interest, we must also emphasize some significant differences between the two systems.

1. Cu(II)-ribonuclease does not require O_2 to exhibit visible and near-ultraviolet CD and absorption bands.

2. Some of the CD bands already shown to be in the same position are in reality of different sign. In arthropod hemocyanins the 550- to 600-nm band is positive, unlike both the molluscan hemocyanins and Cu(II)-ribonuclease. The hemocyanin 305- to 310-nm bands have always been found to be negative.

3. Molluscan hemocyanins all show an additional positive CD band above 700 nm.

In view of the nature of the similarities, it would seem reasonable to suggest that the copper-binding site in hemocyanins is tetradentate, involving at least 1 histidine residue. We may further infer from the common appearance of presumed charge-transfer bands involving the peptide backbone that at least a portion of the remaining ligands is peptide nitrogens. One unique aspect of these metalloprotein metal-binding sites is then seen to be a conformation that allows sufficient peptide proton labilization to achieve tetradentate binding at neutral pH.

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